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THE EFFECT OF L-TRIIODOTHYRONINE ON THE GROWTH AND DEVELOPMENT OF EMBRYONIC CHICK LIMB-BONES IN TISSUE CULTURE

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In the intact animal both hypo- and hyperthyroidism produce characteristic changes in the skeleton which have been observed both clinically and under experimental conditions; this work has been briefly considered in our previous paper (Fell & Mellanby, 1955). The experiments of Kaltenbach (1953) have shown that in amphibian embryos thyroxine can affect the developing limb-skeleton directly, since the implantation into one limb-rudiment of pellets containing thyroxine caused the bones to grow and ossify more rapidly than in the corresponding untreated limb of the same individual. It seemed likely that if the action of L-thyroxine on the skeletal tissues of warm-blooded animals were direct, then isolated bone rudiments in culture would be affected by thyroid active principles; as described in a previous publication (Fell & Mellanby, 1955) this proved to be true.

The object of the present work was to study the effect of triiodothyronine (TIT) on bone rudiments cultivated *in vitro*, and to compare the action and potency of this hormone with those of thyroxine (T). TIT was discovered by Gross & Leblond (1951), and was identified in human plasma (1952) and synthesized (1953*a*) by Gross & Pitt-Rivers; in animal experiments it has usually proved by different criteria to be much more potent than thyroxine. Its antigoitrogenic action on rats treated with thiouracil is several times as great as that of T (Gross & Pitt-Rivers, 1953*b*; Heming & Holtkamp, 1953; Tomich & Woollett, 1954). In thyroidectomized rats its calorigenic effect is not less than 3·5 times that of T (Heming & Holtkamp, 1953), and in maintaining the growth-rate of such animals it is about 5 times as active (Gross & Pitt-Rivers, 1953*b*). The potency of TIT is 3·7–7·5 times that of T in increasing

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† Sir Edward Mellanby died before this paper was written, but he left notes on certain aspects of the work and some of these have been included. H. B. F.

the oxygen consumption of normal rats (Tomich & Woollett, 1954) and about 4·5 (Tomich & Woollett, 1954) or 5–10 times (Anderson, 1954) that of T in producing anoxia in mice. In normal human subjects, TIT is about 10 times as active as T in depressing the uptake of radioactive iodine by the thyroid (Starr & Liebhold-Shoeck, 1953), and 5–10 times as effective in suppressing the release of radioactive iodine from the thyroid of rats (Anderson, 1954). In accelerating amphibian metamorphosis, TIT is 8 times as active as T for tadpoles of *Rana catesbiana* (Bruice, Winzler & Kharasch, 1954), and 3·8 times for those of *Rana clamitans* (Shellabarger & Godwin, 1954).

That TIT is an effective therapeutic agent in cases of human myxoedema was first shown by Gross, Pitt-Rivers & Trotter (1952). Asper, Selenkow & Plamodon (1953) found that the maximal rise in oxygen consumption in myxoedematous patients treated with TIT was 5–10 times that induced by an equimolar amount of T. Rawson, Rall, Pearson, Robbins, Poppell & West (1953) recorded that a single dose of TIT given to such a patient had a more rapid but also a more short-lived effect than the same quantity of T. Giving daily doses, Lerman (1953) found TIT to be 4–5 times as active as T in the treatment of myxoedema.

Although in general TIT has been found to be several times more potent than T, there are exceptions to this. Heming & Holtkamp (1953) found that the calorigenic effects of TIT and T in intact, as opposed to thyroidectomized, rats were the same. Recently Brown-Grant (1955) compared the relative abilities of T and TIT to suppress the secretion of the pituitary thyrotrophic hormone in the rabbit and found a ratio of only 1 : 1·9 (weight for weight) for the relative potency of T : TIT.

The results described in the present paper show that in the same concentration, TIT has a much more drastic action than T on skeletal rudiments grown in culture, though qualitatively the effect of the two substances appears to be the same.

MATERIALS AND METHODS

Material. The cartilaginous rudiments of the femur, tibia, humerus, ulna and radius were removed from 6- to 7-day-old chick embryos. As in our previous study (Fell & Mellanby, 1955) the chicks were divided into groups designated 2, 3, 4 according to the stage of differentiation attained by the limb-bone rudiments, since embryos of the same age differ considerably in their degree of development. In group 3 the rudiments consisted of very young cartilage with little matrix and no chondroblastic hypertrophy in the shafts; in most of the chicks the articular surfaces had already developed, but in a few embryos which really belonged to group 2 the position of the joints was only faintly indicated. In group 4, the characteristic zones of the cartilaginous long-bones, viz. small-celled epiphyses, proliferative zones of flattened cells and mid-diaphysial region of hypertrophic cells, had begun to differentiate, the matrix was fairly plentiful and the joints were distinct.

Methods. The rudiments were grown in a mixture of 3 parts of fowl plasma and 1 part of embryo extract. The extract was made from 13- to 14-day-old embryos with Tyrode solution containing 1 % glucose, so that the final clot contained about 0·25 % glucose. The explants were transplanted

at 2-day intervals and the experiments were terminated after 8 days' growth. Further details of the culture method are given in previous publications (Fell & Mellanby, 1952, 1955).

The L-triiodothyronine and L-thyroxine were dissolved in 0·1% Na_2CO_3 and added to the plasma in the amounts required, the same quantity of Na_2CO_3 without the hormone being introduced into the control medium (C). To prepare the medium most commonly used, 1 mg TIT was dissolved in 32 ml. of a 0·1% solution of Na_2CO_3; 0·03 ml. of this solution was diluted with 4·5 ml. plasma, giving a concentration of 20·8 μg TIT in 100 ml. plasma. After 1 part of embryo extract had been added to 3 parts of plasma, the final medium contained about 15·6 μg/100 ml. of added TIT; unless otherwise stated, this was the concentration used in the following experiments and was the same as that of T in the medium designated X_2 in our previous paper (Fell & Mellanby, 1955). Thyroxine was introduced into the plasma in the same way.

In some experiments the explants were drawn at 2-day intervals with the aid of a camera lucida and their lengths were measured in the manner previously described (Fell & Mellanby, 1955); in other experiments the rudiments were measured directly by means of a micrometer eyepiece.

For histological study, explants were fixed in 3% acetic Zenker's solution for 30–45 min, embedded in paraffin wax and serially sectioned; the blocks were oriented on the microtome with great care, so that each series contained an approximately median longitudinal section. The sections were stained with Delafield's haematoxylin, differentiated in 10% acetic alcohol, and after being washed for a few minutes in running tap water, were counterstained with chromotrop 2R in absolute alcohol.

In several experiments the length of the shaft from the borders of the two epiphyses, and that of the zone of hypertrophic cartilage, were measured in an approximately median longitudinal section of each explant; by subtracting the length of the hypertrophic zone from that of the diaphysis, the total length of the two zones of flattened cells was obtained (see Pl. 1). The lengths of the proliferative and hypertrophic regions in each explant in TIT medium were expressed as percentages of the corresponding figures for the rudiment in control medium (C) and the results are plotted in Text-fig. 4. Such measurements are inevitably very crude, but the figures obtained demonstrated certain gross differences between the rudiments.

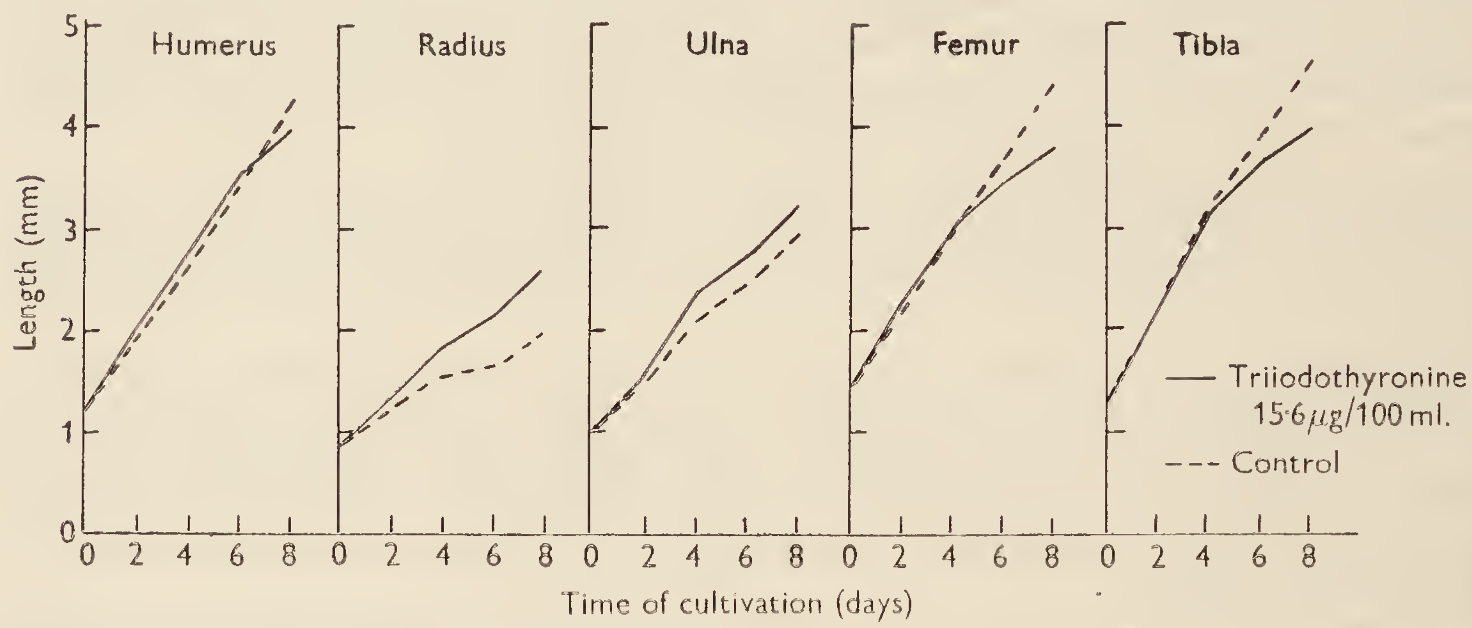
RESULTS

The effects of L-*triiodothyronine* (*TIT*) *on growth and development*

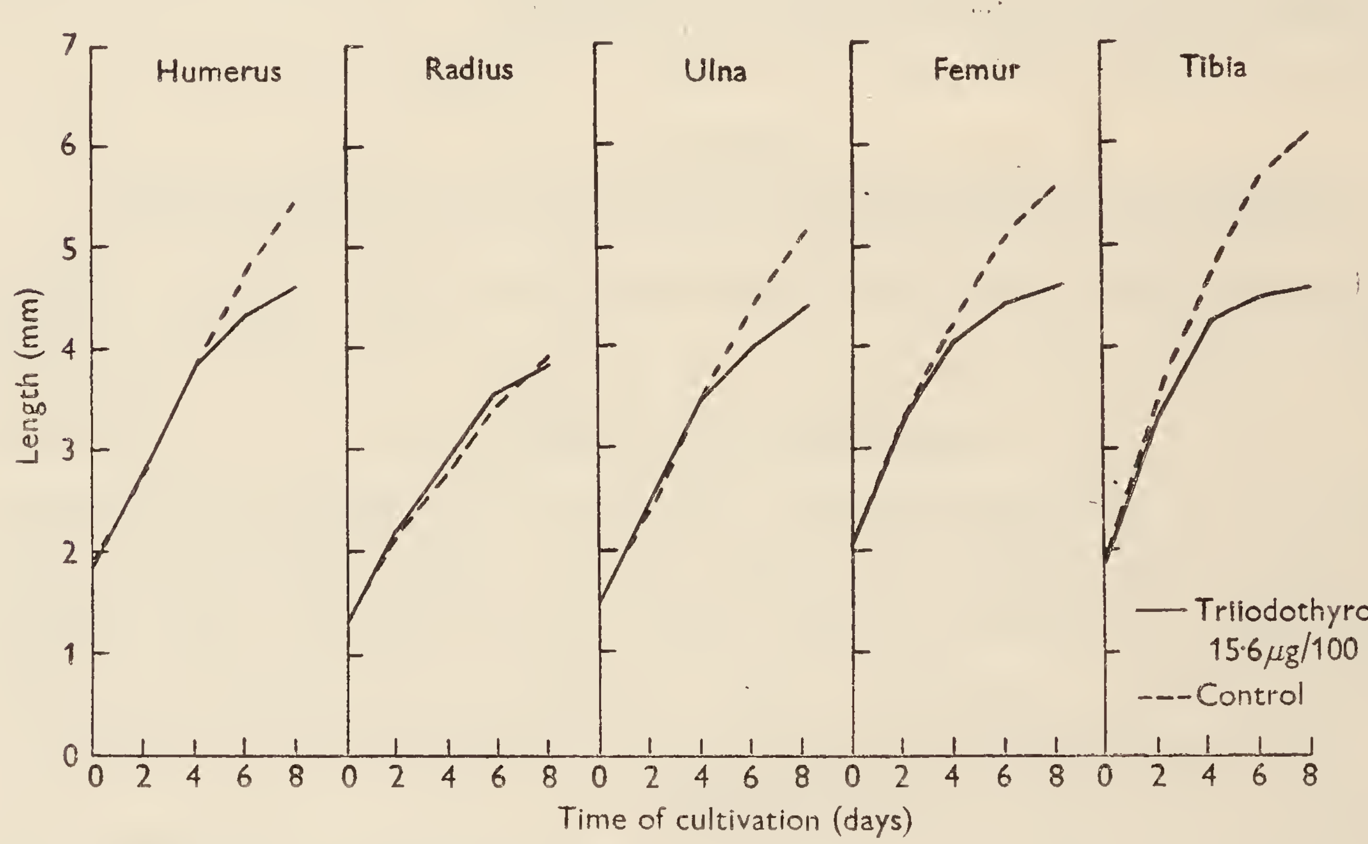
In these experiments, one of each pair of long-bone rudiments was explanted in control medium (C) and the other in medium (TIT) containing 15·6 μg/100 ml. of added hormone. Twenty pairs of explants from four embryos of group 3, and the same number from four chicks of group 4, were cultivated for 4 days, then fixed and sectioned. Forty pairs of rudiments from group 3 (eight embryos) and twenty pairs from group 4 were cultivated for 8 days before fixation; in addition, another forty pairs from group 4 were measured at 2-day intervals for a period of 8 days, but were not histologically studied. As the rudiments of group 3 responded rather differently from those of group 4, the two series will be described separately.

Rudiments of group 3. The curves of the average growth (Text-figs. 1, 3) show that with the same dose of TIT there was little difference in the rate of growth of the femur, tibia and humerus during the first few days, but that the growth of all three was inhibited from the 6th day. In the radius and ulna growth was stimulated throughout the 8 days.

The serial camera lucida drawings of the explants demonstrated that in the femur, tibia and humerus the reduced growth-rate in TIT was due entirely to shortening of the diaphysis, and that in all five rudiments the epiphyses grew more rapidly in TIT than in C. In the femur, tibia and humerus the diminished diaphysial growth was often noticeable by the 4th day, but at this stage there



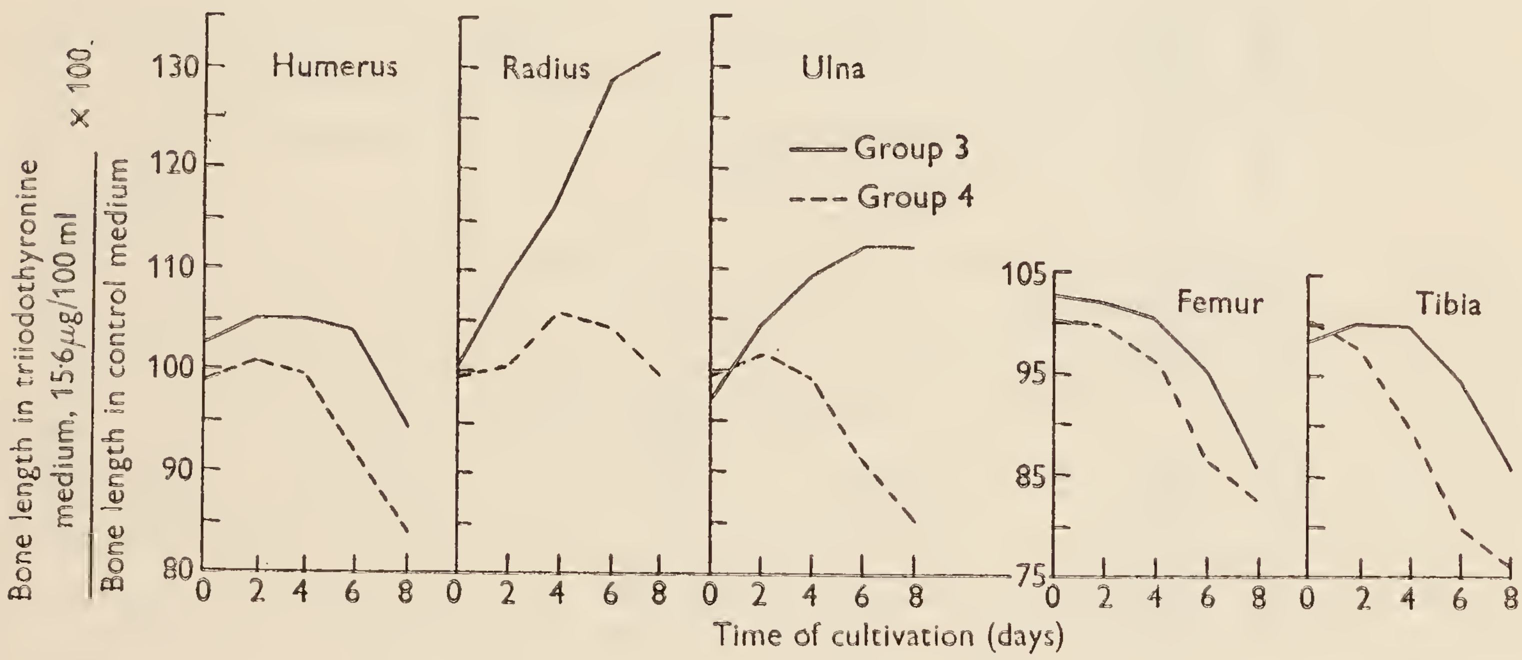
Text-fig. 1. Group 3: graphs showing the average growth in length of the paired limb-bone rudiments in control medium (C) and in medium + 15·6 μg TIT/100 ml. respectively; each graph represents twelve pairs of explants for 0, 2 and 4 days, and eight pairs for 6 and 8 days. The growth of the femur, tibia and, to a lesser degree, of the humerus has been diminished by TIT, but that of the radius and ulna has been accelerated.



Text-fig. 2. Group 4: graphs showing the average growth in length of the paired limb-bone rudiments, in control medium and in medium + 15·6 μg TIT/100 ml. respectively; each graph represents sixteen pairs for 0, 2 and 4 days and twelve pairs for 6 and 8 days. There is a very marked depression of the growth-rate in the femur and tibia, a similar but lesser diminution in that of the humerus and ulna, but no effect on the growth of the radius.

was no corresponding decrease in the total length of the rudiment owing to the accelerated growth of the epiphyses.

There was some variation in the type of development displayed by the twelve pairs of ulnae and radii. Five pairs of ulnae and four pairs of radii formed a normal shaft and epiphyses in both media, but in two pairs of ulnae and two pairs of radii only the explant grown in TIT developed a recognizable shaft and epiphyses, while the corresponding rudiments in C failed altogether to differentiate and formed only a small oval nodule of cartilage. In three pairs of radii and two pairs of ulnae, both explants formed an undifferentiated nodule which was much larger in TIT than in C.



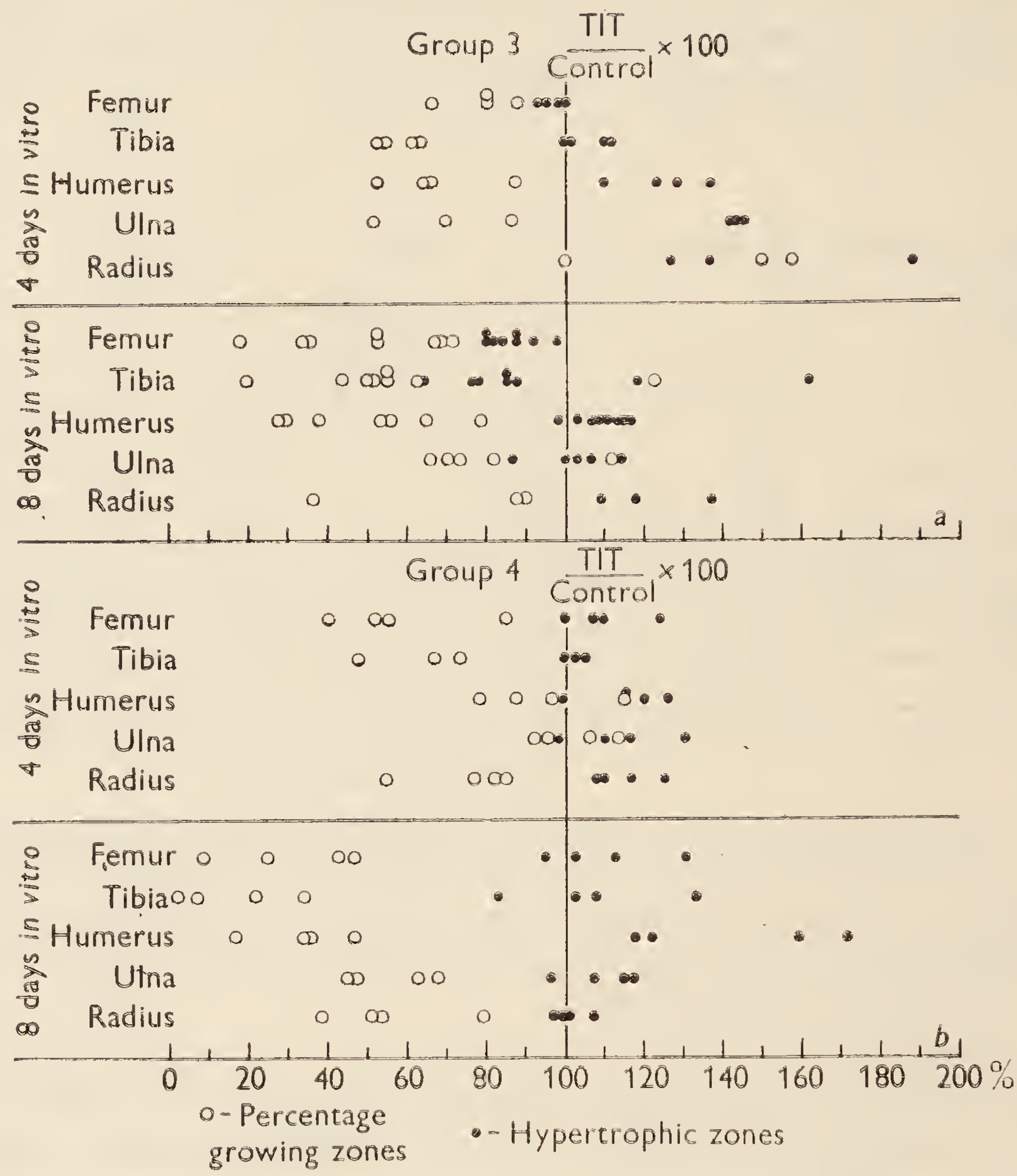
Text-fig. 3. Groups 3 and 4: graphs showing the average rates of growth of the explants in TIT (15·6 μg TIT/100 ml.) relative to that of the corresponding rudiments in control medium. The relative growth-rates of the femur, tibia and humerus are similar in both groups, but in the radius and ulna growth is considerably accelerated by TIT in group 3, but is unaffected (radius) or depressed (ulna) in group 4.

Many of both the wing- and leg-rudiments grown in TIT differed from those in C, in the more abrupt transition between the shaft and the epiphyses; thus in the presence of TIT the epiphyses sometimes jutted sharply from the diaphysis, whereas in the controls the diaphysis expanded gradually to merge with the epiphyses.

All the explants were histologically examined. Sections showed that by the 4th day the epiphyses of all the rudiments were more developed in TIT than in C, as indicated by the larger size of the chondroblasts and more plentiful matrix (Pl. 1, figs. 1, 2; Pl. 2, fig. 5); this accounted for the fact that in TIT as described above, the epiphyses enlarged more rapidly than in C.

It will be seen from Text-fig. 4, that in the *leg-bones* fixed after 4 and 8 days' cultivation respectively (Pl. I, figs. 1, 3), the length of the growing zones, except in one pair, was much less in TIT than in C; this was responsible for the abrupt transition between the epiphyses and the shaft which, as described

above, was often seen in the living explants. The length of the hypertrophic zone was about the same in both media at the 4th day (Text-fig. 4; Pl. 1, fig. 1), but after 8 days it was less in TIT than in the controls except in two pairs of tibiae; the reduction in the hypertrophic region, however, was much less than in the proliferative cartilage. In the youngest hypertrophic cartilage, i.e. that immediately beneath the periosteum and adjoining the growing zones, cellular hypertrophy was more advanced in TIT than in C, but the cells were



Text-fig. 4. Groups 3 and 4: graph showing the lengths of the proliferative and hypertrophic zones in sections of each TIT-treated explant, expressed as percentages of the corresponding figures for the controls in normal medium. In both groups the proliferative zone is considerably shorter than in the controls, while except in the leg-bones of group 3, the hypertrophic zone is usually longer.

separated by narrower partitions of matrix; a similar effect was produced by thyroxine (Fell & Mellanby, 1955). In some of the 8-day leg-bones in both media, there was a varying amount of degeneration in the middle segment of the shaft. In both TIT and C, a fairly thick layer of periosteal bone, often mixed with islets of cartilage, had usually been deposited on the surface of the diaphysial cartilage.

In the *wing-bones*, as in the leg rudiments, the growing zones were shorter

in TIT than in C (Text-fig. 4; Pl. 1, fig. 2; Pl. 2, fig. 6) except in the three pairs of radii measured after 4 days and in one 8-day ulna. The hypertrophic zone of the wing-rudiments (Text-fig. 4; Pl. 1, fig. 2), unlike that of the leg-bones, was longer in TIT, except in one pair of 8-day humeri and two pairs of 8-day ulnae; the increased length of the hypertrophic region was particularly noticeable at the 4th day (Text-fig. 4). The mid-diaphysial degeneration seen in the leg-rudiments also appeared in the wing-bones, where it was more extensive and advanced in TIT than in C.

As recorded above, the degree of anatomical development achieved by the ulnae and radii varied considerably and was associated with a similar variation in histological differentiation. Those explants in TIT or C, which formed a recognizable shaft and epiphyses, had the usual three zones of cartilage, but the oval nodular explants consisted only of small-celled cartilage (Pl. 1, fig. 4). The explants grown in TIT were nearly always more advanced than their controls.

The following conclusions may be drawn from these histological findings. TIT accelerated the differentiation of both the epiphyses and the hypertrophic cartilage, but the stimulatory effect on the latter was considerably greater in the wing-bones, especially the radius, than in the leg-rudiments; the degeneration of the hypertrophic cells also was hastened in the wing-bones but very little, if at all, in the leg rudiments. The proliferative zones were much reduced in width; in the wing-bones, this seemed to be due mainly to the precocious hypertrophy of the cells since the length of the hypertrophic zone was increased, but in the leg-rudiments the hypertrophic zone also was shorter than in the controls, which suggested that in these bones there was a growth-inhibitory effect as well as an acceleration of hypertrophy.

Rudiments of group 4. TIT drastically inhibited the growth of all the rudiments except the radius (Text-figs. 2, 3). The leg-bones were the most affected, and the average growth curves of the femur and tibia in TIT showed earlier and more severe depression than those of group 3. The effect on the humerus was less, but here also diminution of growth in TIT appeared earlier and was greater than in the humeri of group 3. There was a striking difference between the growth of the ulnae in groups 3 and 4; whereas the younger rudiments grew more rapidly in TIT throughout the culture period, the growth of the older ulnae was retarded in this medium. The radii of group 4 at first showed a slight acceleration of growth in TIT though much less than the radii of group 3, but by the 8th day the growth-curve had fallen below that of the controls in normal medium.

The gross anatomical development of the explants in group 4 was similar to that already described for group 3. Histological examination of the explants showed that the response to TIT of the rudiments of group 4, in which hypertrophy was already present at the time of explantation, differed in certain

respects from that of the younger explants in which hypertrophy had not yet appeared. In both groups the epiphysial cartilage was better developed and the growing zones were narrower in TIT than in C (Text-fig. 4; Pl. 2, fig. 7), but after 8 days the diminution of the growing zones was in general greater in group 4 than in group 3 (Text-fig. 4, cf. *a* and *b*); in both groups, however, the reduction was least in the ulna and radius. In group 4, the hypertrophic zone was longer in TIT in most of the rudiments from both leg and wing, whereas in group 3 by the 8th day the hypertrophic zone in the femur and tibia was usually shorter in TIT than in C, but slightly longer in the wing-rudiments.

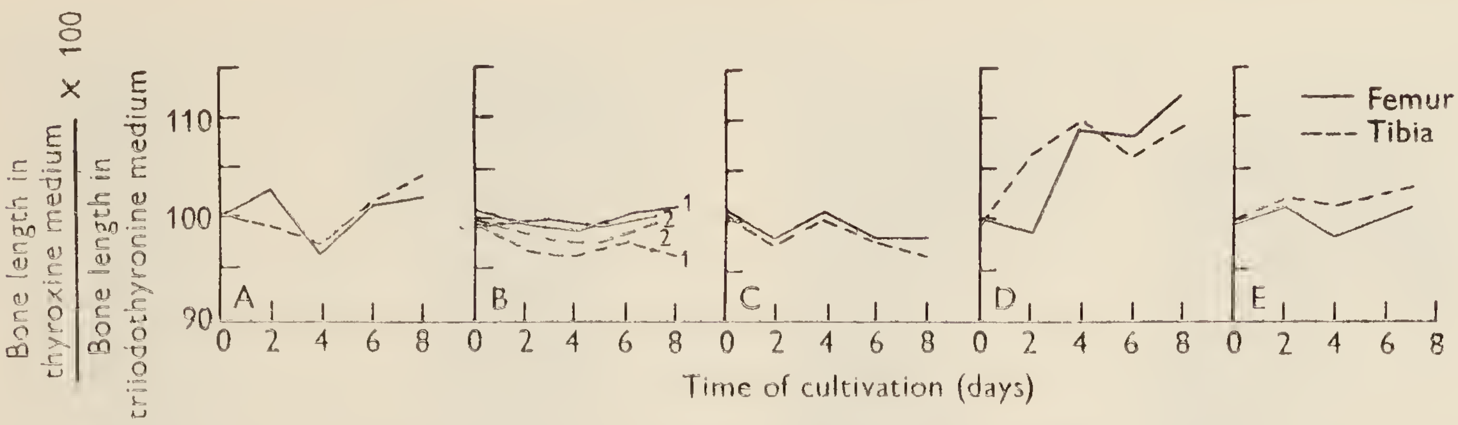
The relative potency of triiodothyronine (TIT) and thyroxine (T)

The relative potency of TIT and T in inhibiting the growth of explanted femora and tibiae was investigated by growing one of each pair of rudiments in medium containing a known concentration of TIT and the other in medium containing a known concentration of T. The lengths of the bones were measured at intervals of 2–3 days. For each comparison of doses 7–9 pairs of femora and the same number of tibiae, all of group 4, were used; their average growth-rate was obtained and was expressed as the ratio $\frac{\text{length in T}}{\text{length in TIT}} \times 100$. The effects of the following doses (μg/100 ml.) were compared (see Text-fig. 5):

(*a*) TIT, 3·9; T, 12·1 (femora, 7 pairs; tibiae, 7 pairs).
(*b*) TIT, 3·9; T, 15·6 (Expt. 1, femora, 7 pairs; tibiae, 7 pairs; Expt. 2, femora, 9 pairs; tibiae, 9 pairs).
(*c*) TIT, 3·9; T, 20·7 (femora, 7 pairs; tibia, 7 pairs).
(*d*) TIT, 15·6; T, 15·6 (femora, 8 pairs; tibiae, 8 pairs).
(*e*) Control experiment: both members of each pair were grown in normal medium (femora, 5 pairs; tibiae, 4 pairs).

It will be seen from the curves in Text-fig. 5, that in the proportion of 3·1:1 (curve A), T reduced growth less than TIT, in a proportion of 5·3:1 (curve C) it reduced it more, and in a proportion of 4:1 (curves B), the effect of the two hormones was about the same. So far as the method allows, therefore, it may be concluded that TIT is about 4 times as potent as T in its growth-inhibitory effect on the embryonic femur and tibia cultivated *in vitro*.

Four pairs of femora and four pairs of tibiae from group 3 and four from group 4 were histologically examined after 8 days' growth; one rudiment from each pair was cultivated in medium containing 15·6 μg/100 ml. of TIT and the other in medium to which the same quantity of T had been added. The proliferative zones were narrower in rudiments grown in TIT than in those grown in T and there was considerably more cell degeneration in the hypertrophic zone, but otherwise there was little difference in histological structure.



Text-fig. 5. Graphs showing the average rates of growth of the femur and tibia on medium containing various concentrations of TIT, relative to that of the corresponding rudiments on medium containing different amounts of T. Concentrations in μg/100 ml. Curve A: T, 12·1; TIT, 3·9. Curve B (two experiments): T, 15·6; TIT, 3·9. Curve C: T, 20·7; TIT, 3·9. Curve D: T, 15·6; TIT: 15·6. Curve E: control: $\frac{\text{Length of right rudiment}}{\text{Length of left rudiment}} \times 100$; both grown in normal medium. In a proportion of 3·1:1 (curves A), T reduced growth less than TIT, in a proportion of 5·3:1 (curves C) it reduced it more, and in a proportion of 4:1 (curves B) the effect of the two hormones was about the same.

DISCUSSION

When the effect of TIT on the bone rudiments is compared with that of T (Fell & Mellanby, 1955), no qualitative histological difference can be detected, but quantitative studies on the relative growth-rates show that TIT is about 4 times as effective as T; a similar relative potency of these two substances has been found in many experiments *in vivo* (see above).

The fundamental action of both TIT and T, in the concentrations used in our experiments, seems to be an acceleration of the normal histological processes of skeletal development, but the effect of both hormones is conditioned by the developmental pattern on which it is superimposed; for this reason the response varies in different regions of the same bone rudiment, in different rudiments from the same chick, and in rudiments at different stages of differentiation. In the chick the epiphyses continue to enlarge throughout embryonic life by the deposition of intercellular material, by some increase in the size of the chondroblasts, and by cell division (Fell, 1925). In the explants, the epiphyses behave in the same way, but under the influence of TIT the formation of matrix and the enlargement of the cells are hastened, so that the epiphysial cartilage grows faster than in the controls in normal medium.

In the shaft, TIT, like T, hastens cellular hypertrophy, so that it spreads abnormally fast into the growing zones; since there is no compensatory increase in mitosis these zones become progressively narrower than in the controls. An essentially similar phenomenon has been reported by Richter (1944) in the testis of hyperthyroidic guinea-pigs; this author describes 'an unbalance of the normal physiologic growth and differential spermatogenic

process resulting in a precocious maturation of the germ cells without a compensatory increase in the spermatogenic growth phase'. The precocious hypertrophy induced by TIT is followed by an earlier cessation of mitosis and of matrix formation, and in the wing-bones by a premature onset of degeneration in the middle of the shaft.

The histological changes produced in the diaphysial cartilage by TIT, like those evoked by T in our earlier experiments, cause a severe inhibition of growth in some, but not in all, of the explanted rudiments. In normal development, the shaft does not differentiate in exactly the same way or at the same rate in all the long bones and if the rudiments are arranged according to the rapidity with which hypertrophy progresses, the order is: tibia, femur, humerus, ulna, radius. From the curves in Text-fig. 3, it is seen that if the five rudiments grown *in vitro* are arranged according to the growth inhibitory effect produced in each by TIT, the order is the same as that of their normal rates of hypertrophy *in vivo*. Thus in both groups of explants, inhibition by TIT is greatest in the tibia, femur and humerus, which differentiate most rapidly in the embryo. On the other hand, in the ulna and radius, which have the slowest rates of hypertrophy *in vivo*, TIT causes, not a depression, but a marked acceleration of growth in group 3. This is mainly due to the fact that whereas the development of the ulna and radius from the younger embryos is usually poor and sometimes arrested in normal medium (see Pl. 1, fig. 4*a*), the stimulatory action of TIT largely overcomes this retardation and consequently the hormone-treated rudiments are larger and better differentiated than their controls (Pl. 1, fig. 4*b*). In the ulnae and radii of group 4, in which hypertrophy had already appeared at the time of explantation, there is little if any retardation in the controls and only very slight stimulation in the TIT-treated explants, followed by a depression of growth which, however, is less than in the two leg bones and the humerus.

So little is known about the metabolic activity of the different bone rudiments in the normal embryo, that it is not possible to offer a satisfactory explanation of the diversity of their response to TIT and T. The results of the present study, however, confirm the view (Fell & Mellanby, 1955) that the effect of the hormones is correlated with the normal rate of diaphysial differentiation in the different bones, as well as with their stage of development when first subjected to the influence of the agents.

SUMMARY

1. The effect of L-triiodothyronine (TIT) on the explanted limb-bone rudiments: femur, tibia, humerus, radius and ulna, of $5\frac{1}{2}$- to 6-day chick embryos was studied and compared with that of L-thyroxine (T).

2. In a concentration of $15\cdot6\,\mu$g/100 ml. TIT accelerated the differentiation and growth of the epiphyses in all five rudiments.

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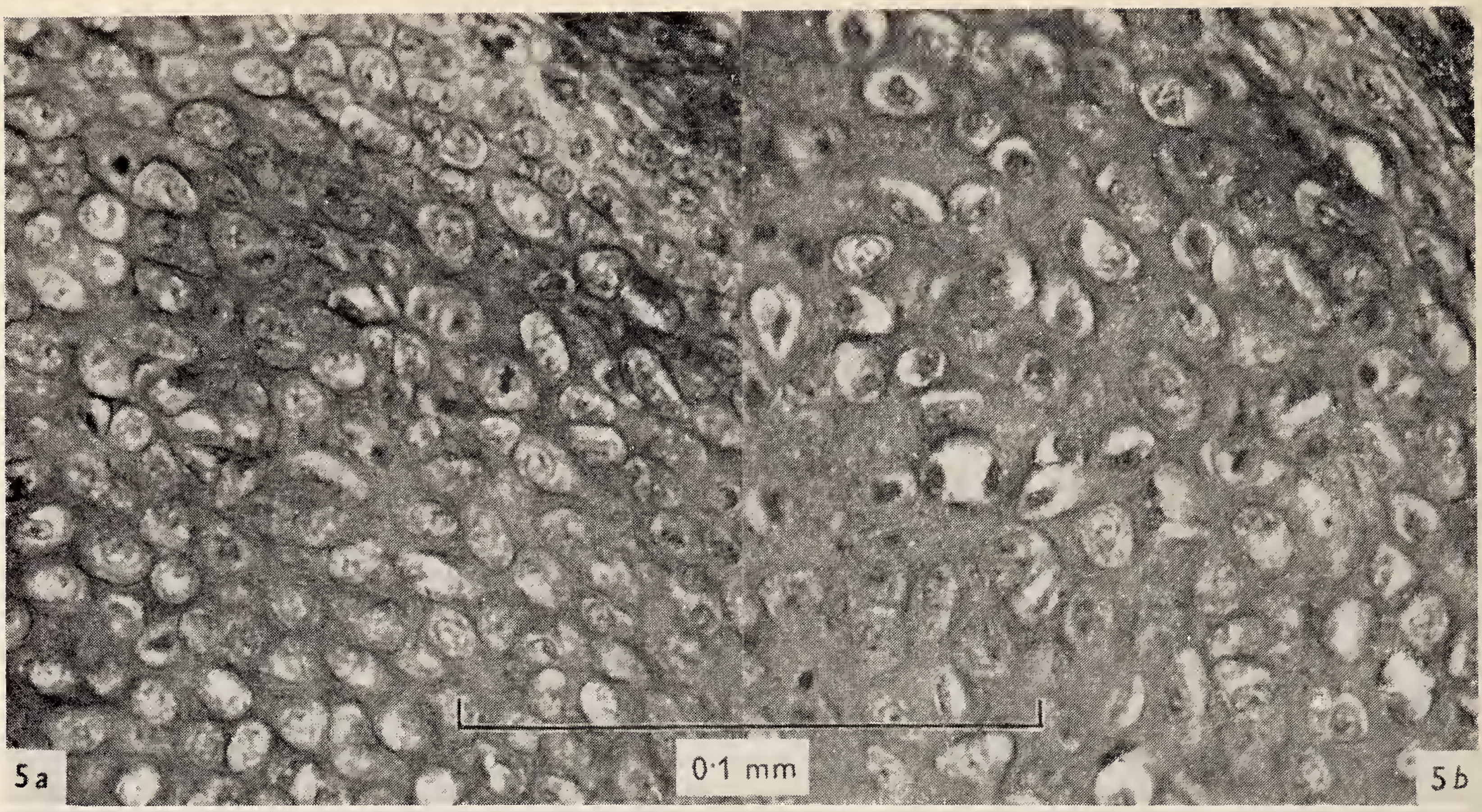
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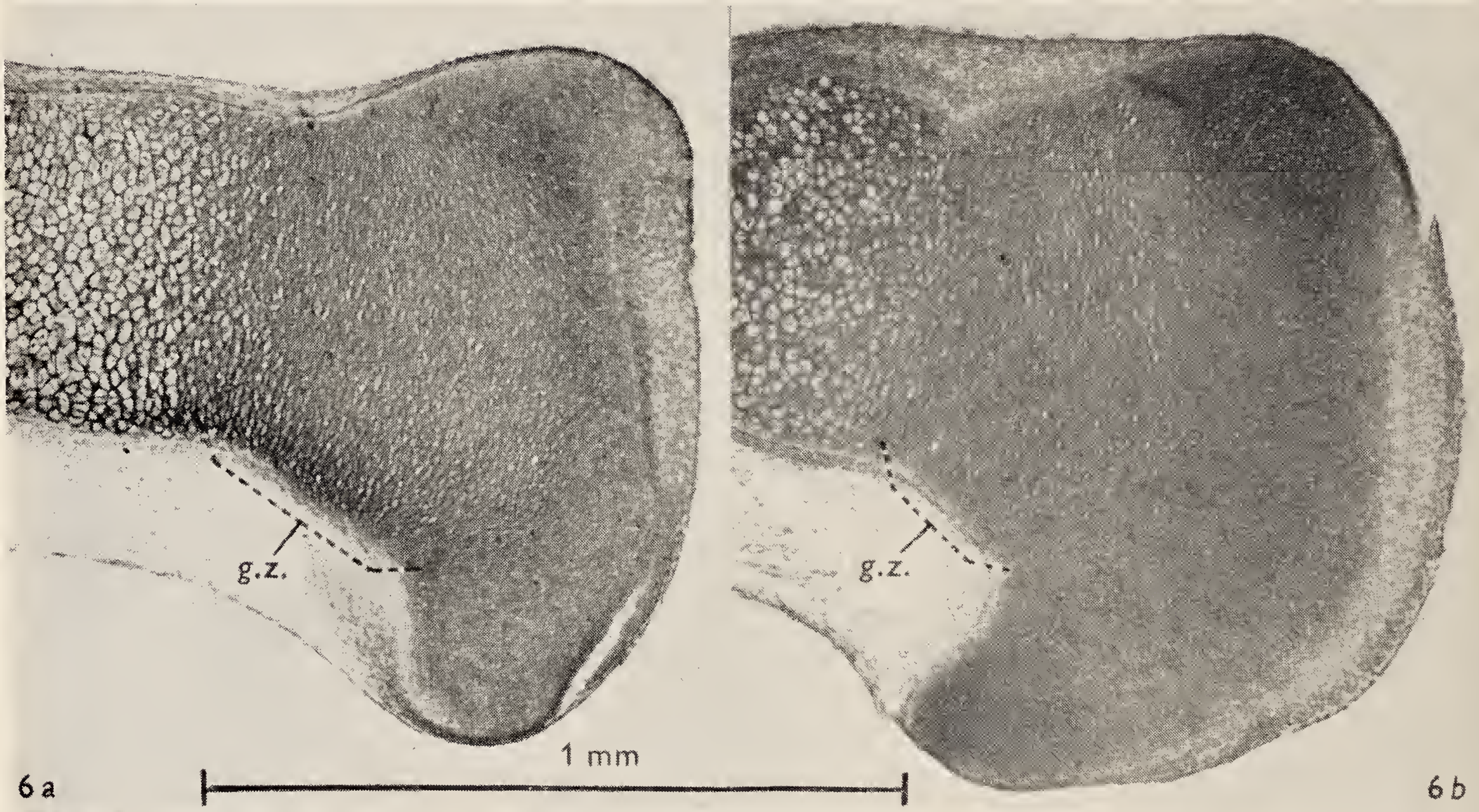
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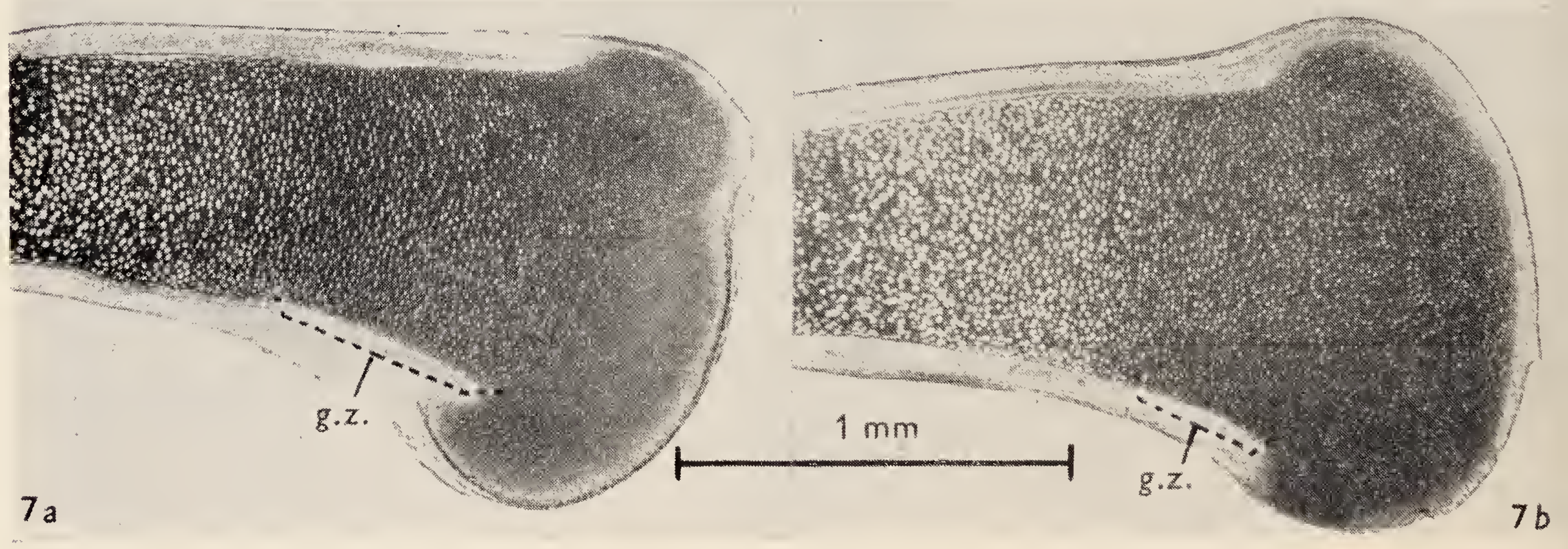
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3. This concentration of TIT induced precocious hypertrophy of the diaphysial cartilage cells, followed by an earlier cessation of mitosis and of matrix formation and usually by a premature onset of degeneration in the middle region of the shaft.

4. Hypertrophy spread abnormally fast into the growing zones of the TIT-treated explants; since there was no compensatory increase in mitosis, these zones became progressively narrower than in the controls.

5. The effect of TIT on the growth-rates of the five rudiments seemed to be correlated with their normal rates of diaphysial differentiation and with their stage of development at the beginning of the experiment. Thus the histological changes produced in the shaft by TIT severely inhibited growth in the rapidly developing femur and tibia and less severely in the humerus; in the more slowly differentiating radius and ulna, growth was stimulated in explants from the younger embryos and slightly retarded in those from older chicks.

6. The histological effects of TIT were qualitatively indistinguishable from those of T, but in a given concentration the inhibitory effect of TIT on the growth rates of the femora and tibiae was about 4 times that of T.

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EXPLANATION OF PLATES

Photographs by Mr V. C. Norfield, Strangeways Research Laboratory.

All the sections were stained with Delafield's haematoxylin and chromotrop 2R. *ep.*, epiphysis; *g.z.*, growing zone; *z.h.c.*, zone of hypertrophic cells.

Plate 1

Fig. 1. *a*: femur from an embryo of group 3, grown for 4 days in control medium (C); *b*: opposite femur from the same chick grown in medium containing TIT 15·6 μg/100 ml. The epiphyses are larger and the growing zones narrower in C than in TIT; the hypertrophic zones are about the same size.

Fig. 2. Humeri from the same embryo as the femora in Fig. 1 after 4 days' growth (*a*) in C, (*b*) in TIT. The epiphyses are larger, the hypertrophic zone slightly longer and better developed and the growing zones narrower in TIT than in C.

Fig. 3. Femora from an embryo of group 3, after 8 days' growth (*a*) in C, (*b*) in TIT. Note the great reduction of the growing zones in TIT.

Fig. 4. Radii from an embryo of group 3, after 8 days' cultivation (*a*) in C, (*b*) in TIT. Differentiation has been suppressed in C and the explant has produced only a nodule of unossified, small-celled cartilage, whereas the radius in TIT has formed hypertrophic cartilage, growing zones and epiphyses in the normal way.

Plate 2

Fig. 5. Comparable areas in the proximal epiphyses of the femora shown in Fig. 1. (*a*) in C, (*b*) in TIT. The cartilage is better developed in TIT than in C; the cells are larger and the matrix is more profuse.

Fig. 6. The proximal ends of the humeri shown in Pl. 1, fig. 2 (*a*) in C, (*b*) in TIT. The epiphysis is larger and the growing zone is narrower in TIT than in C.

Fig. 7. The proximal ends of the femora from an embryo of group 4, after 8 days' cultivation (*a*) in C, (*b*) in TIT. The growing zone is greatly reduced in TIT.